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Apolipoprotein A5, a Crucial Determinant of Plasma Triglyceride Levels, is Highly Responsive to PPARα Activators

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Running title: Gene Regulation of Apolipoprotein A5 by PPARα

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SUMMARY

The recently discovered *APOA5* gene has been shown in humans and mice to be important in determining plasma triglycerides (TG) levels, a major cardiovascular disease risk factor. apoAV represents the first described apolipoprotein where over-expression lowers triglyceride levels. Since fibrates represent a commonly used therapy for lowering plasma triglycerides in humans, we investigated their ability to modulate *APOA5* gene expression and consequently influence plasma TG levels. Human primary hepatocytes treated with Wy 14,643 or fenofibrate displayed a strong induction of *APOA5* mRNA. Deletion and mutagenesis analyses of the proximal *APOA5* promoter firmly demonstrate the presence of a functional PPAR response element. These findings demonstrate that *APOA5* is a highly responsive PPARα target gene and support its role as a major mediator for how fibrates reduce plasma triglycerides in humans.

Abbreviations : *APOA5* : the human gene (italic) ; *Apoa5* : the mouse gene (italic) ; apoAV : the human protein ; apoav : the mouse protein

INTRODUCTION

Coronary heart disease (CHD) continues to be a major cause of morbidity and mortality worldwide. Several epidemiological studies established that, in addition to elevated low density lipoprotein and reduced high density lipoprotein level, elevated triglycerides (TG) constitute an independent risk factor for CHD (1,2). In addition, hypertriglyceridemia is often associated with the metabolic syndrome that characterizes diabetes and obesity (3,4). Therefore, the identification of factors or genes affecting triglyceride metabolism is of significant medical importance for the correction of hypertriglyceridemia and associated-CHD.

Apolipoproteins play a determinant role in lipoprotein metabolism and in lipid homeostasis. More specifically, the *APOA1/C3/A4* apolipoprotein gene cluster is tightly linked to plasma lipid profiles. Indeed, mutations in members of this cluster have been shown to cause severe dyslipidemia and heightened atherosclerosis susceptibility (5-8). A comparative genomic characterization of the *APOA1/C3/A4* gene cluster flanking regions led to the recent identification of a new apolipoprotein gene, apolipoprotein A5 (*APOA5*), present in both mice and humans (9). apoAV shares homology with several apolipoproteins, most prominently apoAIV, and is 368 and 366 amino acids long in mice and human, respectively. This gene appears to be predominantly expressed in the liver and resides on HDL and VLDL lipoprotein particles (9,10).

The properties of apoAV were investigated by creating human *APOA5* transgenic and knockout mice and by searching for associations between human *APOA5* polymorphisms and plasma lipid parameters. The *Apoa5* knockout mice display a 400% increase in plasma triglycerides compared to wild-type mice, while *APOA5* transgenics exhibit triglyceride levels corresponding to one-third of those in control mice. This determinant link between *APOA5* and triglycerides was supported in several separate human studies through the consistent demonstration of associations between *APOA5* single nucleotide polymorphisms and plasma

triglyceride levels (9,11-13). Taken together these mouse and human studies highlight the importance of *APOA5* in the determination of triglyceride plasma levels, and the clinical relevance of identifying factors regulating *APOA5* expression.

Fibrates are hypolipidemic drugs with pleiotropic effects on lipid metabolism including the reduction of plasma triglycerides. Classically, the triglyceride lowering action of fibrates is explained by decreased hepatic secretion of VLDL and an enhancement in plasma triglyceride clearance. Several studies established that this effect is mediated through the induction of lipoprotein lipase expression (14) and down-regulation of *APOC3* expression by fibrates (15). A major means by which fibrates regulate the expression of lipid metabolism-related genes has been shown to be via activation of the peroxisome proliferator-activated receptor alpha (PPARα) (16). Three distinct PPARs (α, β and γ) have been described in different species (17). PPARs are ligand-activated nuclear receptors that dimerize with the retinoid X receptor (RXR) and bind to specific DNA sequence defined as peroxisome proliferator response elements (PPRE). Upon binding, PPARs activate gene transcription.

Given the determinant link between *APOA5* and plasma triglycerides and the widespread use of fibrates in the treatment of dyslipidemia, we investigated whether fibrates can modulate *APOA5* gene expression and consequently influence plasma triglyceride levels. Our studies with human hepatocytes revealed that fibrates dramatically increase *APOA5* expression. Coupled with *in vitro* promoter analysis and the demonstration of a functional *APOA5* PPRE, these data identify fibrates acting via PPARα as a crucial regulator of the new apolipoprotein *APOA5* and suggest a novel clinical mechanism for how PPARα activators can influence triglyceride homeostasis.

EXPERIMENTAL PROCEDURES

Cloning and Construction of Recombinant Plasmids

Exon 1 of the human and mouse *APOA5* gene were determined by examination of the expressed sequence tag database and identification of numerous cDNA clones that terminate at a common 5' basepair in the genome sequence. The four exon structure of *APOA5* is consistent with that of the evolutionarily related apolipoprotein genes (*APOA1,C3,E*). Human *APOA5* promoter fragments (-846/+62, -305/+62, -147/+62) were amplified by PCR using a *APOA5* genomic BAC clone (9) as template and cloned in pGL3 luciferase vector. The followed forward oligonucleotides 5'-AGACCTGTTGGAGGCTATGAATGC-3', 5'-TCTGTTGGGGCCAGCCAG-3', 5'-GGTGCCAGGGAAAGGGCAGG-3' and the reverse oligonucleotide 5'-AATGCCCTCCCTTAGGACTGTGAC-3' primer were used for the PCR reaction. Site-directed mutagenesis (Stratagene) of the *APOA5* promoter was accomplished using the oligonucleotide 5'-AGTGGGAAGCTTAAAGATCATGGGGTT-3' as a mutagenic primer. The human *APOA5*-PPRE (5'-GATCCGGGAAGGTTAAAGGTCATGGGA-3') site oligonucleotide was cloned in tandem repeat into BamHI/BglII sites of pIC20H, digested with HindIII, and subcloned upstream of the thymidine kinase (TK) promoter of pBLCAT4 as described (18).

Cell culture and RNA analysis

Primary human hepatocytes were isolated, maintained and treated with fibrates as described previously (18). RNA preparation and Northern blot hybridizations were performed as described previously (9). The 0.9 kb *APOA5* cDNA probe was amplified by PCR with full-length *APOA5* cDNA as template and the oligonucleotides 5'-GATAATGGCAAGCATGGCTG-3' and 5'-CTGCAGGTAGGTGTCCTGGCGGA-3' and subcloned in pBS-SK+.

Transfections and Transient Expression Assay

Human hepatoma HepG2 cells were maintained and transiently transfected by calcium phosphate coprecipitation as described (18) using 0.3 µg of reporter vector, 30 ng of PPARα expression vector and 30 ng of CMV-β-galactosidase expression vector as a control for transfection efficiency.

Gel Retardation Assay

mPPARα, mRXRα proteins were synthesized *in vitro* using the rabbit reticulocyte lysate systems (Promega). Gel retardation assays were performed as previously described (18).

RESULTS

Human APOA5 gene expression is induced by Fibrate treatment in human primary hepatocytes

To determine whether fibrates can modulate *APOA5* gene expression in humans, we analyzed *APOA5* mRNA levels in primary human hepatocyte upon treatment with fenofibric acid, the active form of fenofibrate, or Wy 14,643, a prototype PPARα agonist. Treatment with fenofibric acid at a concentration (100 µM) (similar to that reached in plasma from treated patients) dramatically induced *APOA5* mRNA levels (about 5 fold increase). We observed a similar effect with Wy 14,643 treatment (Fig. 1). These observations demonstrate that fibrates induce the expression of human *APOA5* suggesting *APOA5* as a new target gene for fibrates.

Gene regulation of APOA5 by fibrates occurs at the transcriptional level

To determine if *APOA5* was directly responsive to PPAR, we examined the proximal *APOA5* promoter for potential PPRE sites (Fig. 2). Upstream of exon 1 a consensus TATAA and

CAATT box were readily apparent, as was a putative PPRE site (-272/-260). In order to delineate the mechanism of regulation of *APOA5* gene expression by fibrates, we performed functional analysis of the *APOA5* promoter. HepG2 cells were transiently transfected with a Luciferase reporter vector driven by different human *APOA5* promoter fragments (from -846/+62, -305/+62, -147/+62). Co-transfection with PPARα strongly stimulated the *APOA5* promoter activity (about 25 fold increase with construct –846/+62; about 40 fold increase with construct –305/+62) in the presence of Wy 14,643 (Fig. 3). Transcriptional activity of the *APOA5* reporter construct was also slightly induced by the addition of Wy 14,643 in the absence of cotransfected PPARα. Deletion analysis of the promoter located a putative PPRE between nucleotides –305 and –147 from the initiation start site. These results support that the gene regulation of *APOA5* by fibrates occurs at the transcriptional level.

APOA5 contains a PPRE that confers responsiveness to PPARa

Transcriptional activation of *APOA5* gene by PPARα suggests the presence of a functional PPRE in the *APOA5* promoter. Sequence analysis revealed the presence of a putative PPRE with a high degree of homology between *APOA5*-PPRE and the PPRE consensus defined for PPARs (19). To assess whether the putative PPRE mediates the PPARα effect, we performed transfection experiments using –305/+62 promoter construct containing a mutated version of the PPRE. The mutation is designed to suppress the binding of PPAR as described (18). Mutation of the PPRE abolished the activation of the *APOA5* promoter by PPARα (Fig. 4).

To prove that the putative *APOA5*-PPRE could function as a PPRE, we cloned it in three oriented copies in front of heterologous promoter thymidine kinase (TK) and challenged with PPARα in HepG2 cells. We found that this site could transmit PPARα activation (about 5.8 fold

increase in the presence of PPARα and Wy 14,643) to the TK promoter (Fig. 5A). We performed electrophoretic mobility shift experiments to examine whether PPAR-RXR heterodimer could bind to the PPRE. Incubation of labelled PPRE oligonucleotide with *in vitro* translated PPARα and RXRα resulted in the formation of a strong retarded complex (Fig 5B). Taken together these results demonstrate that *APOA5* promoter contains a functional PPRE that confers PPARα activator responsiveness.

DISCUSSION

Fibrates are among the most effective agents for lowering plasma triglycerides in humans. In this report, we show that fibrates dramatically affect the expression of the recently identified *APOA5* gene in humans. The previous studies demonstrating that apoAV is selectively produced by the liver and behaves as a regulator of plasma triglyceride levels, coupled with the results of the present study argue in favour of a crucial link between fibrates, *APOA5* and triglyceride metabolism. The up-regulation of human *APOA5* is important regarding of the molecular mechanism of triglycerides homeostasis action of fibrate. Indeed, *APOA5* represents to date the first example of an apolipoprotein whose overexpression leads to a decrease in triglyceride levels (9,20), whereas *APOC3* or other apolipoproteins transgene leads to an increase in triglycerides plasma levels (21-24).

The activation of *APOA5* transcription may be attributed to a PPRE located inside the proximal *APOA5* promoter. Remarkably, this PPRE differs 1 nucleotide from the consensus PPRE [AGGTCA] A [AGGTCA] or DR-1 (direct repeat 1). Binding and functional studies indicates that this PPRE confers a significant PPARα-mediated transactivation. The rate of transcriptional up-

regulation induced by PPARα activation on *APOA5* is among, if not the highest level attained for genes regulated by fibrates studied in human primary hepatocyte model (*APOA2* (18), CPT-1 (25)).

Actually, gene regulation via PPREs is complex. Indeed, the DR-1 structure of the PPRE can integrate different antagonistic actions induced by nuclear receptors such as RXR, RAR (26), HNF4, ARP-1 (or COUP-TF) (27) depending on the sequence of the regulatory element and the context of the promoter. The *APOC3* gene harbours one DR-1 that can bind PPAR/RXR heterodimer, but its promoter responds to PPAR only in non-hepatocyte cells (26), whereas HNF4 (28) or RXR induce transactivation (26). Fibrates down regulate *APOC3* gene expression probably through indirect inhibition of the HNF4 nuclear receptor (29). Alternatively, PPARα activators stimulate Rev-erbα (30) that in turn repress *APOC3* (31). Therefore, *APOA5* is the first triglyceride lowering apolipoprotein gene that is firmly identified as a positive and a direct target gene of PPARα activators.

So far, most of the TG lowering effects have been mainly attributed 1) to the induction of lipoprotein lipase (LPL) gene expression which enhance catabolism of TG-rich particles (14) and 2) to the down-regulation of *APOC3* gene (15) leading to a decreased hepatic VLDL secretion. The exact contribution that alterations in expression of these genes have on plasma triglyceride levels is not known but appears to be dependent on the activation by PPARα. Analysis of the *APOA5* transgenic and knockout mice showed that their changes in plasma triglyceride levels were directly opposite to those previously reported for the *APOC3* knockout and transgenic mice (21,22). The *Apoa5* knockouts display a 400% increase in plasma TG compared with the 30% decrease observed in *Apoc3* knockouts, whereas *APOA5* transgenic showed decreased triglyceride levels compared with the increase reported in *APOC3* transgenics. In addition, overexpression of *APOA5* was accompanied by a lowered apoc3 protein level (9). Therefore, *APOA5* may be defined

as a major determinant of triglyceride levels and from the present study appears to be fibrate responsive through action of PPARα. Based on the magnitude of the effect that altered *APOA5* expression has on plasma triglycerides in mice compared to *APOC3*, *APOA5* may be defined as a potentially major determinant of triglyceride homeostasis. The results of the present study convincingly demonstrate that *APOA5* is a target gene for PPARα activators. This gene regulation of *APOA5*, combinated with the dramatic effects previously shown in *APOA5* transgenic and knockout mice on plasma triglyceride levels suggest a plausible explanation for the ability of PPARα activators to lower plasma TG levels. Modulation of *APOA5* via a PPARα pathway offers a new strategy for intervention designed at correcting hypertriglyceridemia and at limiting TG-associated metabolic disease and cardiovascular risk.

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Footnotes

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FIGURE LEGENDS

Fig. 1. Effect of fenofibric acid and Wy 14,643 on human *APOA5* expression. Human hepatocytes were isolated and treated 24 h with fenofibric acid (100 μM), Wy 14,643 (100 μM) or vehicle (DMSO, dimethylsulfoxide) as indicated. Total RNA (10 μg) was subjected to Northern blot analysis using human *APOA5* cDNA (top panel) or human acidic ribosomal phosphoprotein 36B4 (bottom panel) cDNA probes.

Fig. 2 Sequence upstream of the putative *APOA5* promoter. Exon 1 is depicted by a white box with the start site of transcription indicated by +1. Consensus sites for a CAAT and TATAA box are bold and underlined, as well as a predicted PPRE-DR1 site (-272/-260).

Fig. 3. Delineation of the PPRE in the human *APOA5* promoter. HepG2 cells were transfected with different human *APOA5* promoter fragment reporter plasmids (-846/+62), (-305/+62), (-147/+62) cloned into Luciferase reporter vector. Cells were cotransfected with empty vector pCMV or pCMV-mPPARα and incubated with Wy 14,643 (1μM) (black bars) or vehicle (DMSO, white bars) for 48 h. Luciferase activity is expressed as means ± SD. Luc: luciferase.

Fig. 4. Identification of the *APOA5*-PPRE by mutation analysis. HepG2 cells were transfected with human APOA5 promoter reporter construct (-305/+62) or with a construct containing a mutation (cross) of the putative PPRE. Cells were cotransfected with empty vector pCMV or pCMV-mPPARα and incubated with Wy 14,643 (1μM) (black bars) or vehicle (DMSO, white bars). Luciferase activity is expressed as means ± SD. Luc: luciferase.

Fig. 5. ***APOA5*****-PPRE transmits activation to the TK promoter and binds PPARα.** A, HepG2 cells were transfected with *APOA5*-PPRE(x3) in front of a TK promoter (pBL-CAT reporter) with empty vector pCMV or pCMV-mPPARα and incubated with Wy 14,643 (1µM) (black bars) or vehicle (DMSO, white bars). CAT activity is expressed as means ± SD. B, gel retardation assays were performed with radio-labeled oligonucleotides in the presence of *in vitro* translated mPPARα and mRXRα. CAT: chloramphenicol acyl transferase. Lys : unprogramed rabbit reticulocyte lysate.

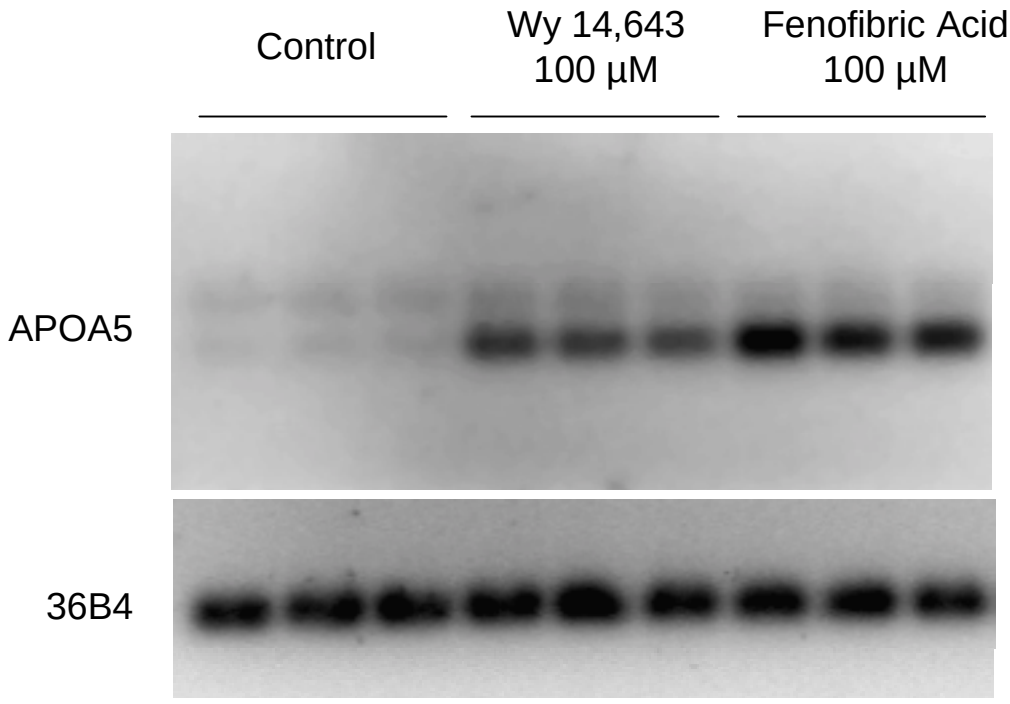

Figure 1

-846

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CAGAGAAGATCTGAGCATTTGGGCTTGCTCTCCTCAGAGGCCCTGCGAGTGGAG

TTCAGCTTTTCCTCATGGGGCAAATCTTACTTTCGCTCCAGTTCCTGGGGCTCA

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-272 -260

CTGTTGGTGGGCCAGCCAGCAGGTCAGTGGGA**AGGTTAAAGGTCA**TGGGGTTTG

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CTTCTCTACTGGGGCAGCCCCTGATACCAGGGCACTCATTAACCCTCTGGGTGC

CAGGGAAAGGGCAGGAGGTGAGTGCTGGGAGGCAGCTGAGGTCAACTTCTTTTG

AACTTCCACGTGGTATTTACTCAGAG**CAATT**GGTGCCAGAGGCTCAGGGCCCTG

-31 +1

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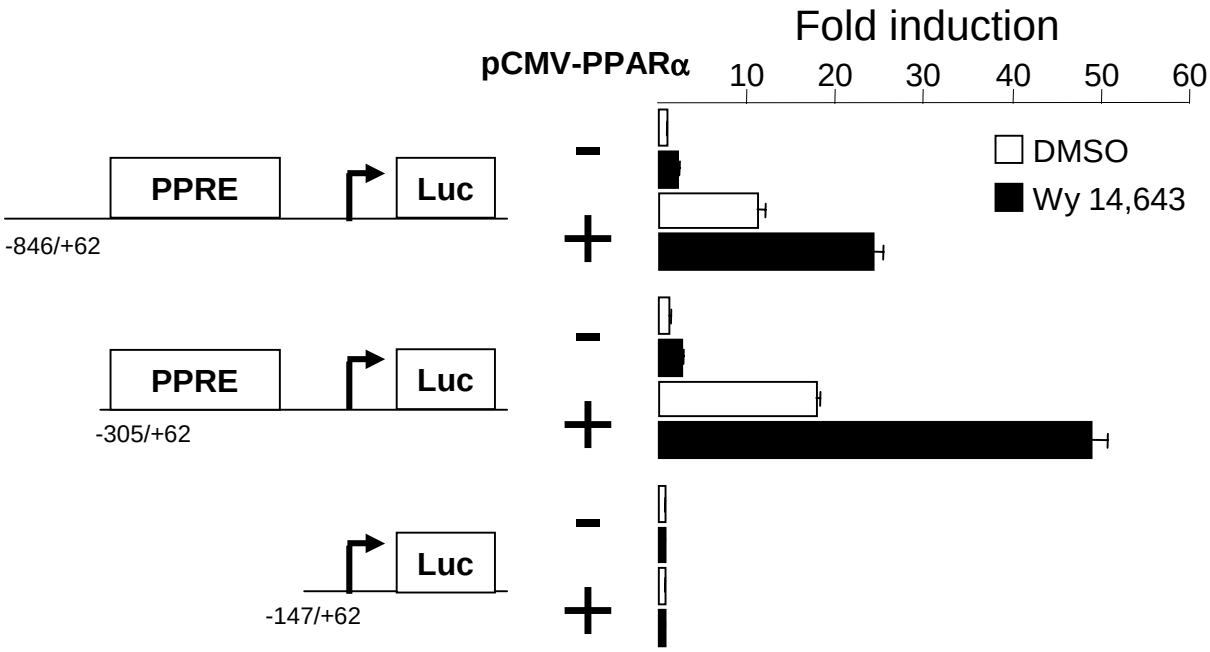
Figure 2


Fold induction
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10
20
30
40
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DMSO
Wy 14,643
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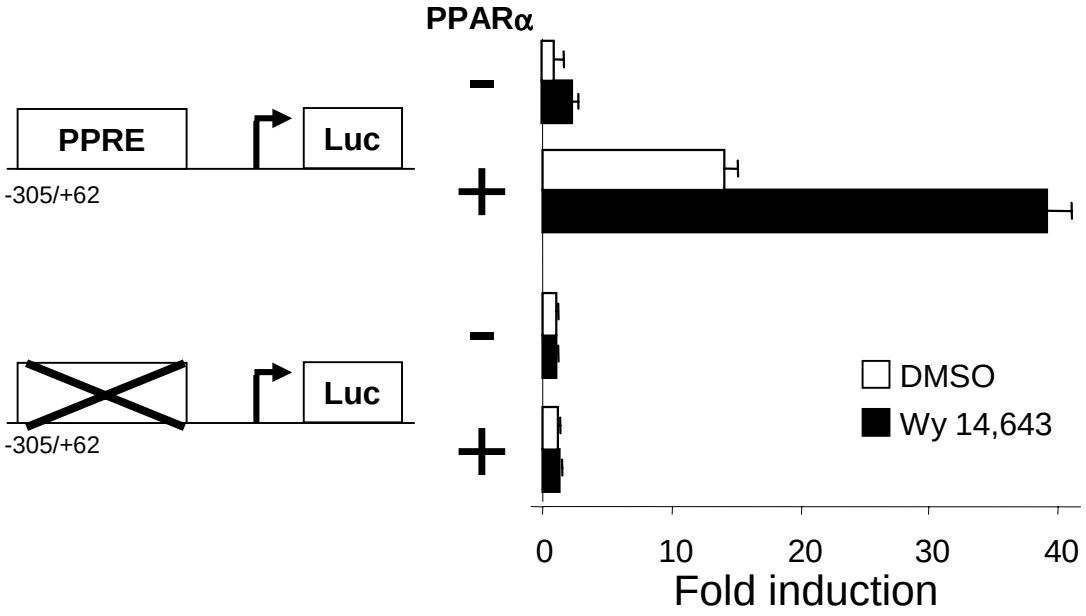
Figure 3


PPARα
PPRE
Luc
-305/+62
Luc
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DMSO
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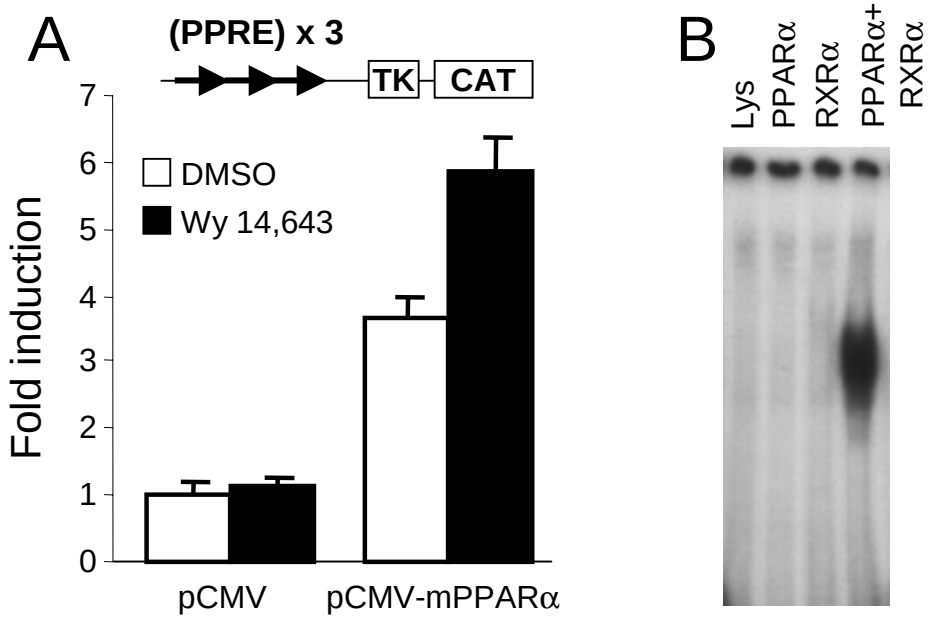
40
Fold induction

Figure 4


A
(PPRE) x 3
TK
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Fold induction
7
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DMSO
Wy 14,643
pCMV
pCMV-mPPARα
B
Lys
PPARα
RXRα
PPARα+
RXRα

Figure 5